AD_____

Award Number: W81XWH-10-1-0491

TITLE: M Current-Based Therapies for Nerve Agent Seizures

PRINCIPAL INVESTIGATOR: Jaideep Kapur, M.D. Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Department of Neurology
Charlottesville, VA 22908

REPORT DATE: July 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| July 2011 | Annual | 1 July 2010 – 30 June 2011 |

**4. TITLE AND SUBTITLE**

M Current-Based Therapies for Nerve Agent Seizures

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-10-1-0491

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Jaideep Kapur, M.D. Ph.D.
Jian Li Sun
Marko Todorovic
E-Mail: jk8t@virginia.edu

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

University of Virginia
Department of Neurology
Charlottesville, VA 22908

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

This is a proposal to develop novel, mechanism-based therapies for the treatment of organophosphate (OP) nerve agent-induced seizures. OP nerve agents, such as sarin, soman and VX act by central and peripheral cholinesterase inhibition and enhance cholinergic transmission. In experimental animals, aknown nerve agents produce convulsive seizures and status epilepticus within minutes of exposure. Proposed time table for the Year 1was to a) Obtain regulatory approval for use of animals and obtain drugs. b) Complete experiments 1.1 and & 1.2, 1.3. Start flupirtine part of experiment 3.1, 3.2 and 3.3. Briefly, we have we have

**15. SUBJECT TERMS**
Seizures, status epilepticus Cholinergic, M Current, Synaptoic transmission

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 18 | 19b. TELEPHONE NUMBER *(include area code)* |

## Table of Contents

**Page**

## Introduction:

This is a proposal to develop novel, mechanism-based therapies for the treatment of organophosphate (OP) nerve agent-induced seizures. The primary goal of this proposal is to test two interrelated **hypotheses**: 1) Cholinergic nerve agents cause neuronal hyper-excitability by inhibiting M/KCNQ2/3 potassium channels. 2) Cholinergic seizures can be blocked by drugs that can open potassium channels mediating M currents. These hypotheses will be tested by accomplishing three aims. **Aim 1)** To test whether modulation of M channel modulators (antagonists and openers) glutamatergic transmission on CA1 pyramidal neurons and dentate granule cells. **Aim 2)** To characterize the effects of M current enhancers on excitabtility and bursting of CA1 pyramidal neurons. **Aim 3)** To test anticonvulsant action of three M-Channel openers in cholinergic overstimulation-induced status epilepticus.

## Body:

Proposed time table for the Year 1was to a) Obtain regulatory approval for use of animals and obtain drugs. b) Complete experiments 1.1 and & 1.2, 1.3. Start flupirtine part of experiment 3.1, 3.2 and 3.3. We have obtained IACUC, and ACURO approvals of the animal care protocol. We have obtained the drugs flupirtine, retigabine, XE 991 and other drugs necessary to complete the study. Dr. Jianli Sun (post-doctoral fellow) and Marko Todorovic (technician) have been working on the project.

EXPERIMENT 1.1: CHARACTERIZE THE EFFECT OF M CHANNEL ANTAGONISTS (ON SPONTANEOUS EPSCS.

### *Methods:*

*Slice preparation*: All studies were performed according to protocols approved by the University of Virginia Animal Use and Care committee and ACURO protocol. A total of forty-nine rats were used. Adult male Sprague–Dawley rats were anesthetized with isoflurane before

decapitation and followed by quick removal of the brain, which was sectioned to 300 μm using a Leica VT 1200 (Leica Microsystems, Wetzlar, Germany) slicer in ice cold oxygenated slicing solution. The solution contained the following (in mM):120 sucrose, 65.5 NaCl, 2 KCl, 1.1 $KH_2PO_4$, 25 $NaHCO_3$, 10 d-glucose, 1 $CaCl_2$, and 5 $MgSO_4$. The slices were incubated at 32 °C at least one hour in oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 127 NaCl, 2 KCl, 1.1 $KH_2PO_4$, 25.7 $NaHCO_3$, 10 d-glucose, 2 $CaCl_2$, and 1.5 $MgSO_4$; osmolarity was 290–300 in the chamber. Then slices were transferred to the recording chamber on the stage of an Olympus Optical BX51 microscope (Olympus, Tokyo, Japan). All chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise.

*Whole cell recording*: Whole cell patch-clamp recordings were performed under infrared differential interference contrast microscopy (Olympus) with a ×40 water-immersion objective to visually identify cells. Slices were continuously superfused with ACSF solution saturated with 95% $O_2$– 5% $CO_2$ at room temperature. Patch electrodes (final resistances, 3–5 MΩ) were pulled from borosilicate glass (Sutter Instruments, Novato, CA) on a horizontal Flaming-Brown microelectrode puller (Model P-97, Sutter Instruments). For voltage-clamp recording, electrode tips were filled with a filtered internal recording solution consisting of (in mM) 117.5 $CsMeSO_4$, 10 2-hydroxyethyl] piperazine-*N*-[2-ethansulfonic acid] (HEPES), 0.3 *N*-[and glycol-bis (a-aminoethyl ether) *N,N,N,N*-tetraacetic acid (EGTA), 15.5 CsCl, 1.0 $MgCl_2$, pH 7.3 (with CsOH); osmolarity was 310 mosM. The electrode shank contained (in mM) 4 ATP $Mg_2^+$ salt, 0.3 GTP $Na^+$ salt, and 5 QX-314. For current-clamp recording, the pipette solution contained the following (in mM): 135 K-gluconate, 2.5 NaCl, 10 HEPES, 0.5 EGTA, 4.0 MgATP, 0.4 NaGTP, 0.1 $CaCl_2$, pH 7.3, 310 mOsm.

Neurons were voltage clamped to −60 mV with PC-505B amplifier (Warner Instruments, Hamden, CT). Electrode capacitance was electronically compensated. Access resistance was continuously monitored, and if the series resistance increased by 25% at any time, then the

recording was terminated. Currents were filtered at 2k Hz, digitized using a Digidata 1322 digitizer (Molecular Devices, Sunnyvale, CA), and acquired using Clampex 10.2 software (Molecular Devices).

Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded from CA1 pyramidal neurons after blocking the $GABA_A$ receptor with the antagonist picrotoxin (50 μM). In preliminary experiments, a combination of 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX) and 2-amino-5-phosphonovaleric acid (APV) blocked all EPSCs. Miniature EPSCs (mEPSCs) were recorded by blocking action potentials with 1μM tetrodotoxin (TTX, Alomone labs, Jerusalem, Israel). All drugs were bath-applied by a peristaltic pump.

*Data analysis*

The off-line digitized data were analyzed with MiniAnalysis (Synaptosoft, Decatur, GA) and Clampfit 10.2 (Molecular Devices). To detect sEPSCs and mEPSCs, a detection threshold three times root mean square (RMS) of baseline noise was used. After detection, frequency and peak amplitude of EPSCs were analyzed for individual neurons. Each detected event in the 20- to 30-min recording was visually inspected to remove false detections. The Kolmogorov-Smirnov (K-S) test was used to compare amplitudes and interevent intervals for continuously recorded EPSCs. Event frequency and amplitudes were compared using a Student's *t*-test. Data values were expressed as the means ± SE unless noted otherwise. The *P* values represent the results of Student's *t*-test analysis, with $p < 0.05$ indicating the level of significance. Drug application data were analyzed using a paired *t*-test.

***Results:***

The role of M channels in Schaffer-collateral CA1 pyramidal synapses was studied by recording sEPSCs from CA1 pyramidal neurons. After recording sEPSCs for 10 min, M-channel blocker XE991 (10 μM) was applied for 10 min. The frequency of sEPSCs increased in

response to XE991 but their amplitude remained unchanged. After 10 min baseline recording, the mean frequency of sEPSCs was 0.11± 0.03 Hz (n = 7) and the mean amplitude of sEPSCs was 16.91 ± 0.92 pA (n = 7). With 10 min of XE991 (10 μM) application, the frequency was increased to 0.30 ± 0.01 Hz (n = 7) and amplitude was16.65 ± 0.80 pA (n = 7), after 10 min normal saline washout, the mean frequency and amplitude were 0.34 ± 0.02 Hz and 17.52 ± 0.80 pA (n = 7). XE991 increased the frequency of sEPSC by 147.56 ± 18.21 % (n = 7, $p <$ 0.01), but did not have significant effect on sEPSC amplitude (n = 7, $p$ = 0.50) (Fig. 1A,B). The cumulative frequency plots of sEPSC inter-event intervals and cumulative amplitude were analyzed by the K-S test. XE991 caused a significant left shift in inter-event intervals (Fig. 1) but has no shift effect on amplitude of sEPSCs (Fig. 1).

Experiment 1.2: To characterize the effect of M current antagonists on miniature excitatory postsynaptic currents (mEPSCs).

To study whether XE991 has effect on action potential independent glutamatergic synaptic transmission in CA1 pyramidal neuron, its effect on mEPSCs was studied using the same time course as of sEPSC studies. XE991 (10 μM) increased mEPSCs frequency by 82.21 ± 7.36 % (baseline 0.13 ± 0.02 Hz, XE991 0.22 ± 0.03 Hz, n = 8, $p < 0.01$), but had no effect on its amplitude (baseline 17.70 ± 1.26 pA, XE991 18.10 ± 1.27 pA, n = 8, $p$ = 0.30) (Fig. 2). XE991 caused a significant left shift in mEPSCs interevent intervals but had no shift effect on amplitude ( Fig 2).

The effect of XE991 on mEPSC frequency was confirmed by another drug that blocks M-channels. linopirdine (10 μM) increased mEPSCs frequency by 68.22 ± 6.05 % (baseline 0.12 ± 0.01 Hz, linopirdine 0.21 ± 0.03 Hz, n = 6, $p < 0.01$), but had no effect on its amplitude (baseline 15.35 ± 1.13 pA, linopirdine 15.78 ± 0.94 pA, n = 6, $p$ = 0.23). Linopirdine also caused a significant left shift in interevent intervals but has no shift effect on amplitude of mEPSCs.

We tested whether opening M-channel would have opposite effect. M channel opener flupritine (20 μM) decreased mEPSCs frequency by 25.50 ± 8.04% compare to baseline (baseline 0.41 ± 0.08 Hz, flupritine 0.27 ± 0.04 Hz, n = 8, $p < 0.01$), but did not change its amplitude (baseline 22.59 ± 1.09 pA, flupirtine 22.73 ± 1.20 pA, n = 8, $p = 0.40$). Flupirtine caused a significant right shift in interevent intervals but has no shift effect on amplitude of mEPSCs.

EXPERIMENT 3.1, 3.2: TO TEST THE ANTICONVULSANT ACTION OF M-CHANNEL OPENER FLUPIRTINE IN CHOLINERGIC OVER-STIMULATION-INDUCED STATUS EPILEPTICUS.

*Whole animals studies ( aim 3)*

In the current study, we examined the efficacy of flupirtine in terminating diazepam-refractory SE induced by cholinergic stimulation. All procedures on animals were performed according to a protocol approved by the institutional Animal Care and Use Committee and ACURO. Adult male Sprague-Dawley rats (Taconic) weighing 250-350 g were housed with food and water ad libitum. A bipolar insulated stainless steel electrode was implanted stereotaxically, under ketamine/xylazine anesthesia, in the over the cortex. The assembly was secured to the skull with dental acrylic, as previously described. After a 5-7 day recovery period, the rats were administered 3 mmol/kg lithium chloride intraperitoneally (i.p.) twenty hours later, SE was induced by i.p. injection of 50 mg/kg pilocarpine. Thirty minutes prior to pilocarpine administration, 2 mg/kg scopolamine was given to each rat to reduce the peripheral effects of the pilocarpine. .

EEG activity was recorded continuously for at least 18 hrs following drug injection to determine the effect of the drug on prolonged SE. SE was considered terminated when the EEG returned to normal baseline or showed irregular spikes without recurrence of seizures in a subsequent observation period of five hours. Behavioral seizures were considered terminated

when there was cessation of behavioral seizures and resumption of exploratory behavior. In some animals end of behavioral seizures was accompanied by sedation where animal lay still in the cage.

All the animals used in the study demonstrated continuous SE for at least 4 hrs from the time continuous electrographic discharges were observed on EEG , with seizures lasting 12 hrs or more in some animals. Electrographically, all the animals demonstrated continuous SE for at least 4 hrs and the electrographic features of SE were similar to those described previously {Treiman, 1990 70 /id}{Wang, 2009 3871 /id}. Animals exhibited wet dog shakes, facial twitching and automatisms, chewing, staring, hind limb scratching, head bobbing, forelimb clonus, rearing, and rearing and falling with generalized convulsions. Animals spent most of their time exhibiting stage 3-5 seizures, briefly interrupted by stage 2 behaviors.

Treatment was initiated 10 min after continuous electrographic seizures because previous studies demonstrated refractoriness to diazepam at this time point{Wang, 2009 3871 /id}. Behaviorally, this corresponds to a time point 10 minutes after first stage 5 seizure{Martin, 2008 3759 /id}. Five rats, treated with normal saline 10 minutes after continuous electrographic seizure, continued to exhibit continuous electrographic seizures for the next 4 hours ( figure 3, 6) . Refractoriness to diazepam was confirmed in another experiment, five animals were treated with 10 mg/kg diazepam 10 minutes after the onset of continuous electrographic seizure and they continued to have seizures for the next 3 hrs ( figure 6) .

In a previous study of neonatal SE induced by kainic acid induced SE, 50 mg/kg flupirtine controlled SE{Raol, 2009 4039 /id}. . We first tested whether 50 mg/Kg dose of flupirtine would control cholinergic SE and it did not control seizures for 4 hours (figure 4, 6).

We tested whether a combination of diazepam (10 mg/kg) with varying doses of flupirtine would be less toxic, more efficacious and faster acting than flupirtine alone. Animals

were treated with diazepam 10 mg and 25 mg/kg flupirtine; electrographic and behavioral seizures were controlled 1 of 5 animals within 60 minutes (figure 6). we increased the dose of flupirtine to 50 mg/Kg in combination with 10 mg/Kg diazepam, seizures were controlled in 4/5 animals within 60 minutes (figure 5 and 6).

The principal finding of this study is that a combination of diazepam and flupirtine terminates prolonged cholinergic stimulation-induced SE in a synergistic fashion. Diazepam increased the efficacy of flupirtine.

## Key Research Accomplishments:

- We found that M-Channels regulate glutamate release from presynaptic terminals. These channels regulate both action potential dependant and independent release. These M channels are modulated by muscarinic receptors. The nerve agents stimulate muscarinic agents. These findings provide novel insights into mechanisms by which nerve agents and organophosphates cause seizures.

- We found that a combination of diazepam and flupirtine terminates prolonged cholinergic stimulation-induced SE in a synergistic fashion.

## REPORTABLE OUTCOMES

1) A presynaptic action of M-type potassium channels in Schaffer-collateral CA1 pyramidal synapses. Abstract submitted to the Society for Neuroscience, Annual meeting, 2011.

## Conclusions:

These studies demonstrate that M-Channels regulate glutamate release from presynaptic terminals. These channels regulate both action potential dependant and independent release. These findings provide novel insights into mechanisms by which nerve agents and organophosphates cause seizures. These suggest that drug targeting M channels could be effective in terminating SE caused by cholinergic agents. We found that a combination

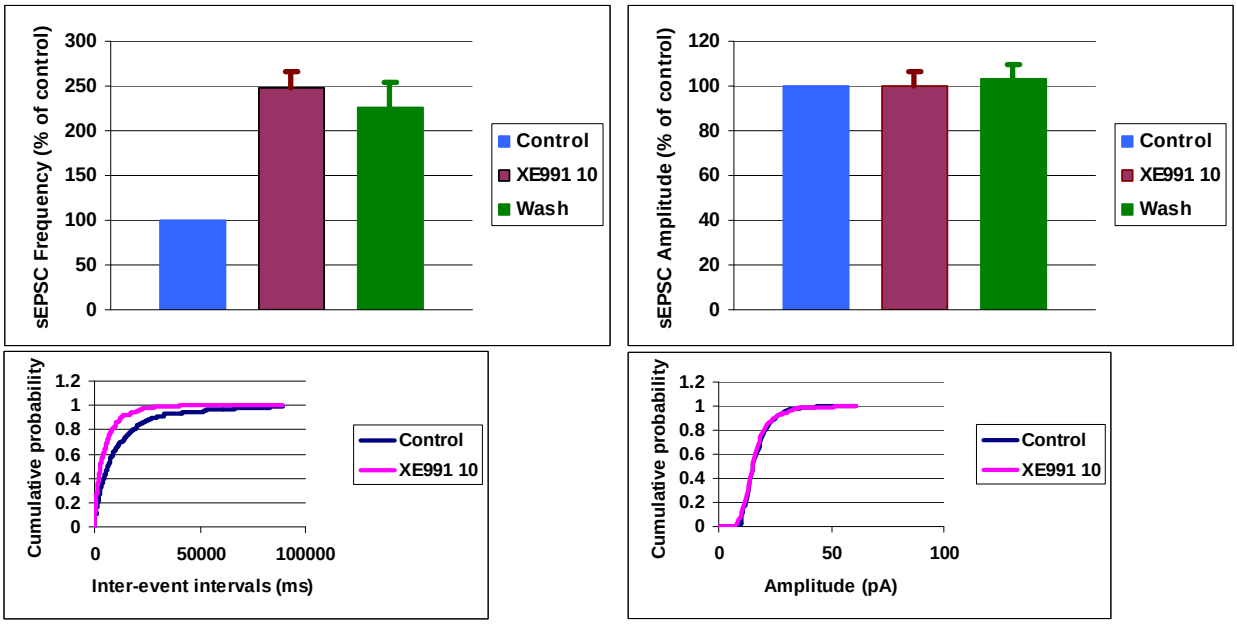
of diazepam and flupirtine terminates prolonged cholinergic stimulation-induced SE in a synergistic fashion.

Future implications: In the future we will address how M channels modulate action potentials and action potential independent release of excitatory transmission.

In the future we need to address whether flupirtine will be effective in terminating organophosphate induced seizures. In the original application, We had proposed using the organophosphate paraoxon, but seizures caused by this agent are easily controlled by diazepam. Seizures caused by another organophosphate (DFP) are diazepam refractory. We will use DFP model to study these seizures.

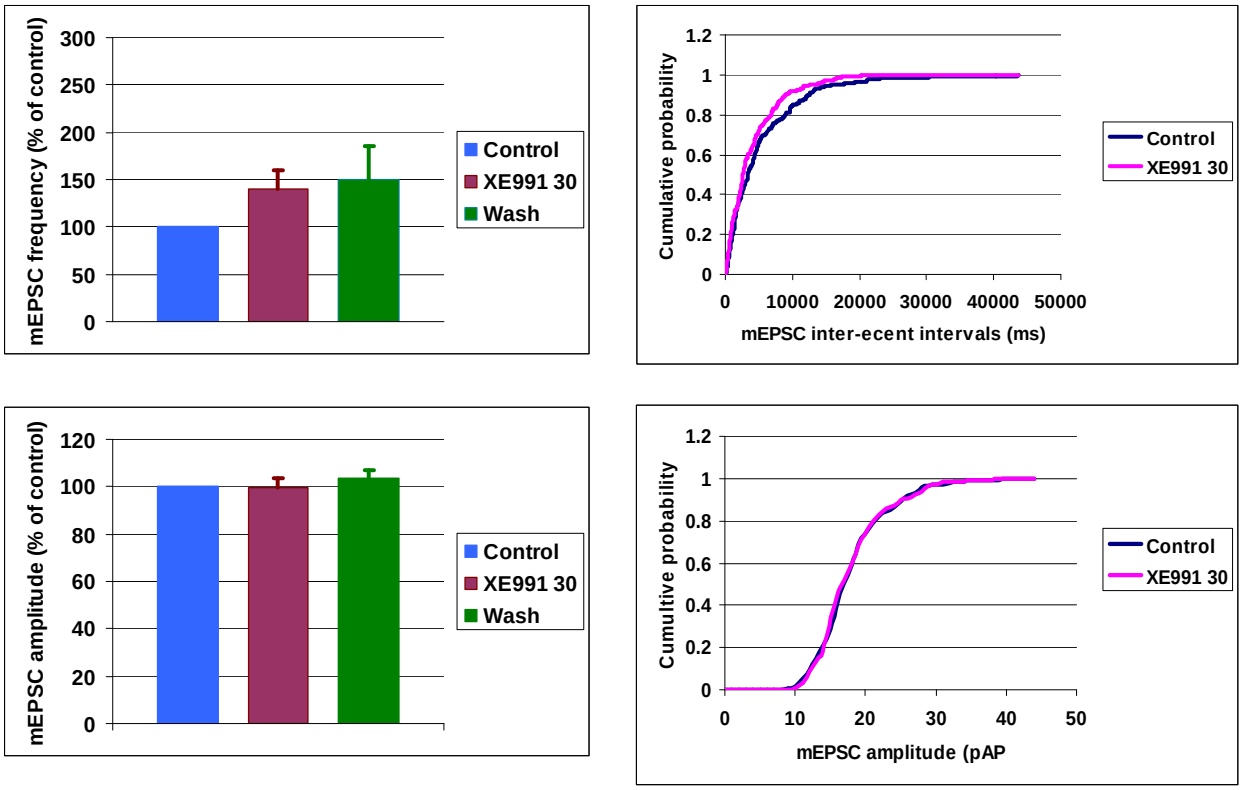
## Appendices

Figure 1




Effect of inhibiting M currents with XE 991 on the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCS).

Figure 2




Effect of inhibiting M currents with XE 991 on the frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCS)

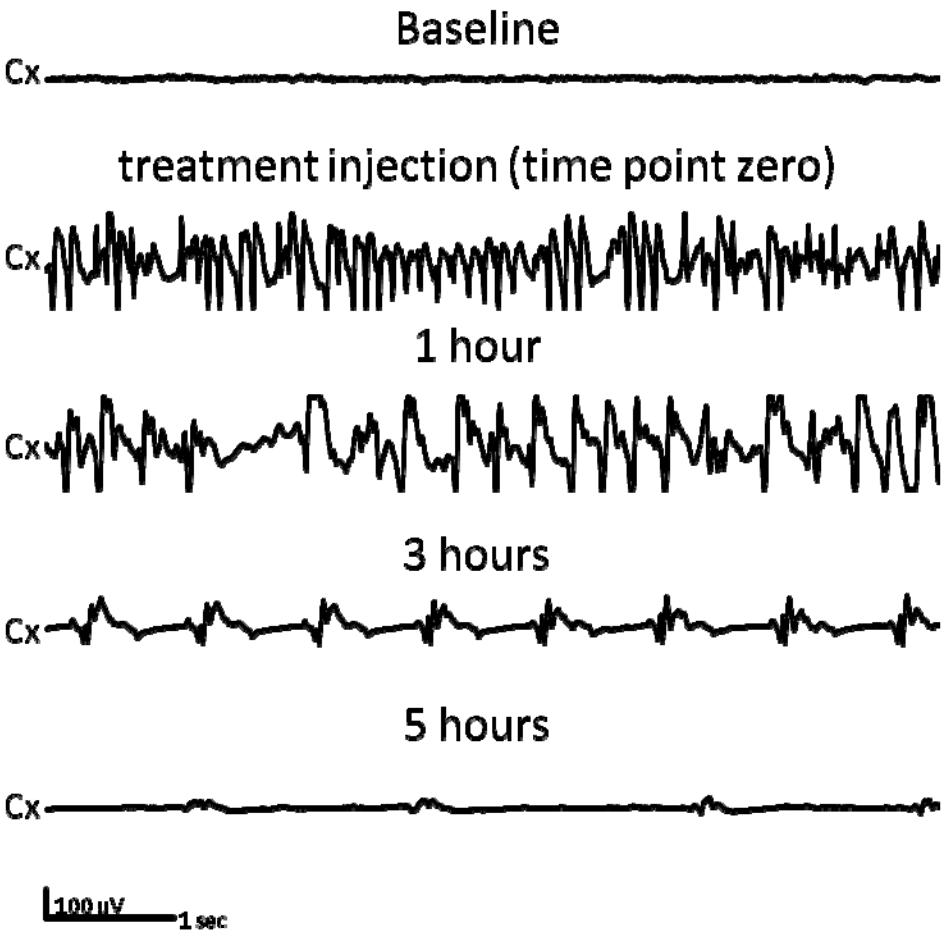



Figure 3. EEG recording from animal in status epilepticus induced by a combination of lithiumand pilocarpine treated with saline (control).

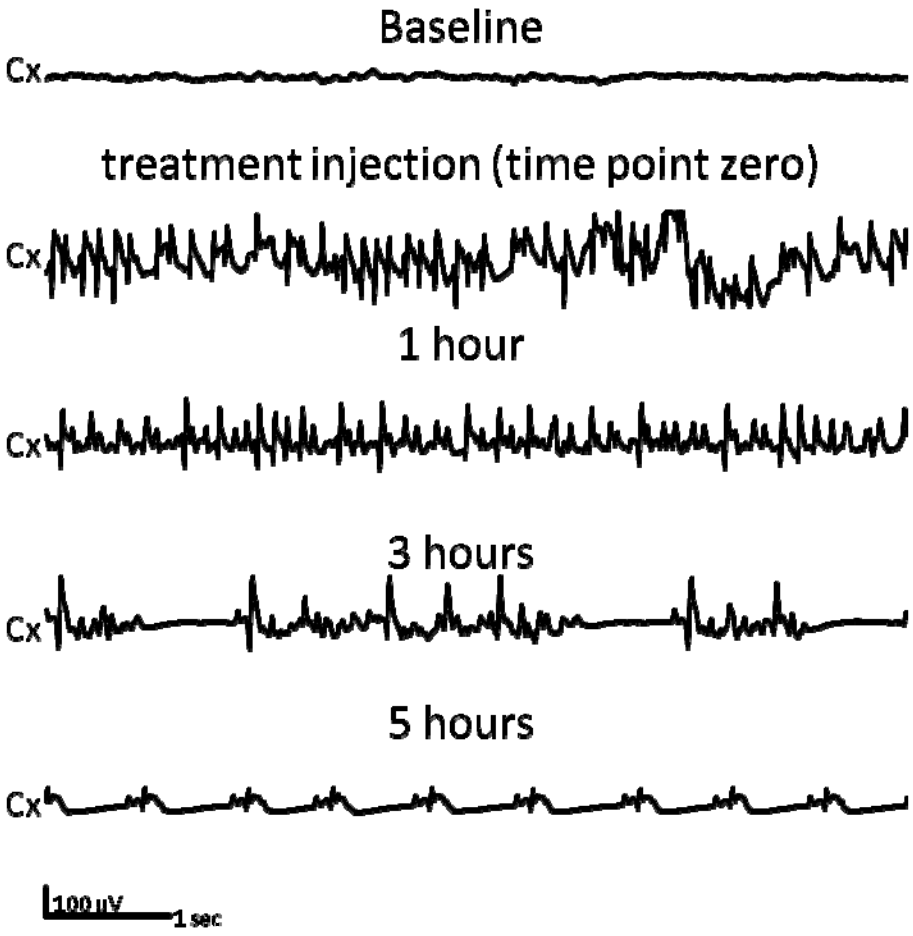



Figure 4. EEG recording from animal in status epilepticus induced by a combination of lithium and pilocarpine treated with 50 mg/Kg flupirtine after the oset of status epilepticus. Note lack of effectiveness, seizures continue despite treatment .

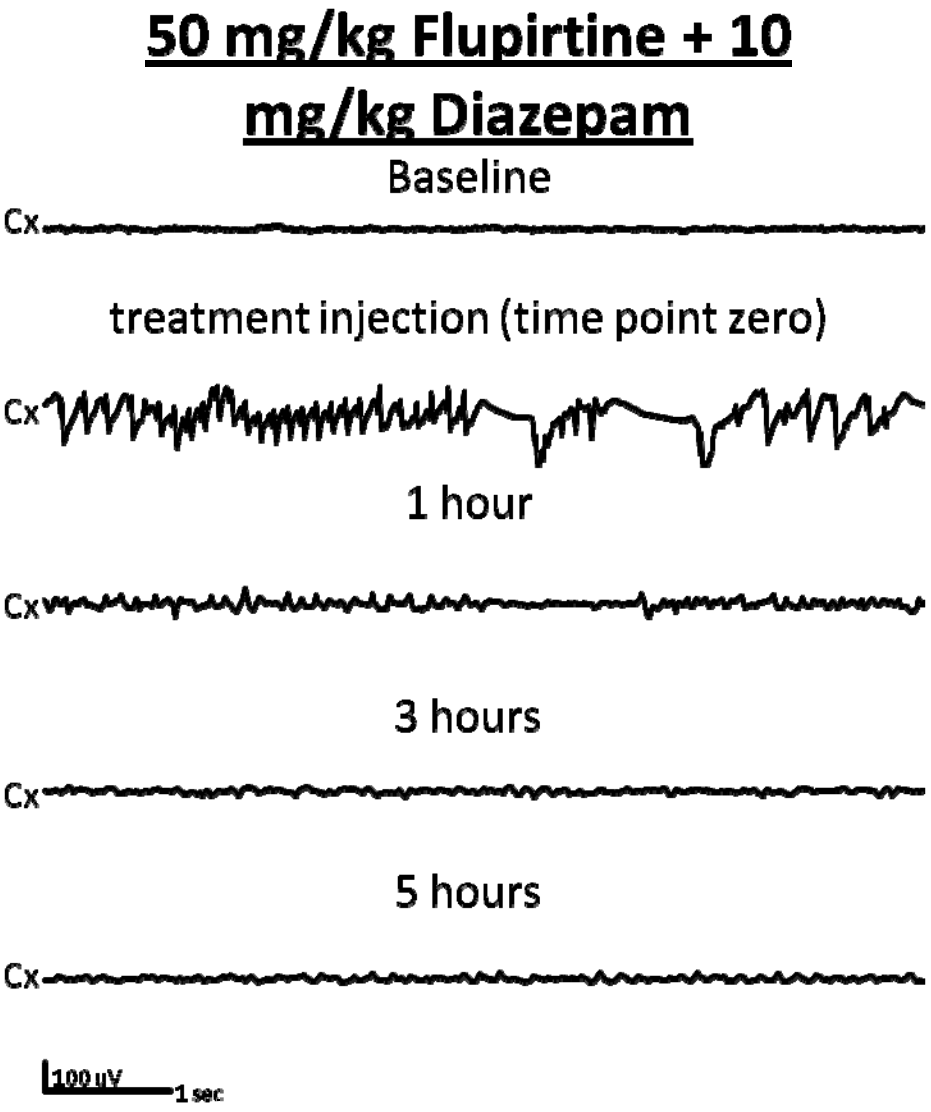



Figure 5. EEG recording from animal in status epilepticus induced by a combination of lithium and pilocarpine treated with 50 mg/Kg flupirtine and 10 mg/Kgafter the onset of status epilepticus. Note lack of effectiveness, seizures continue despite treatment.

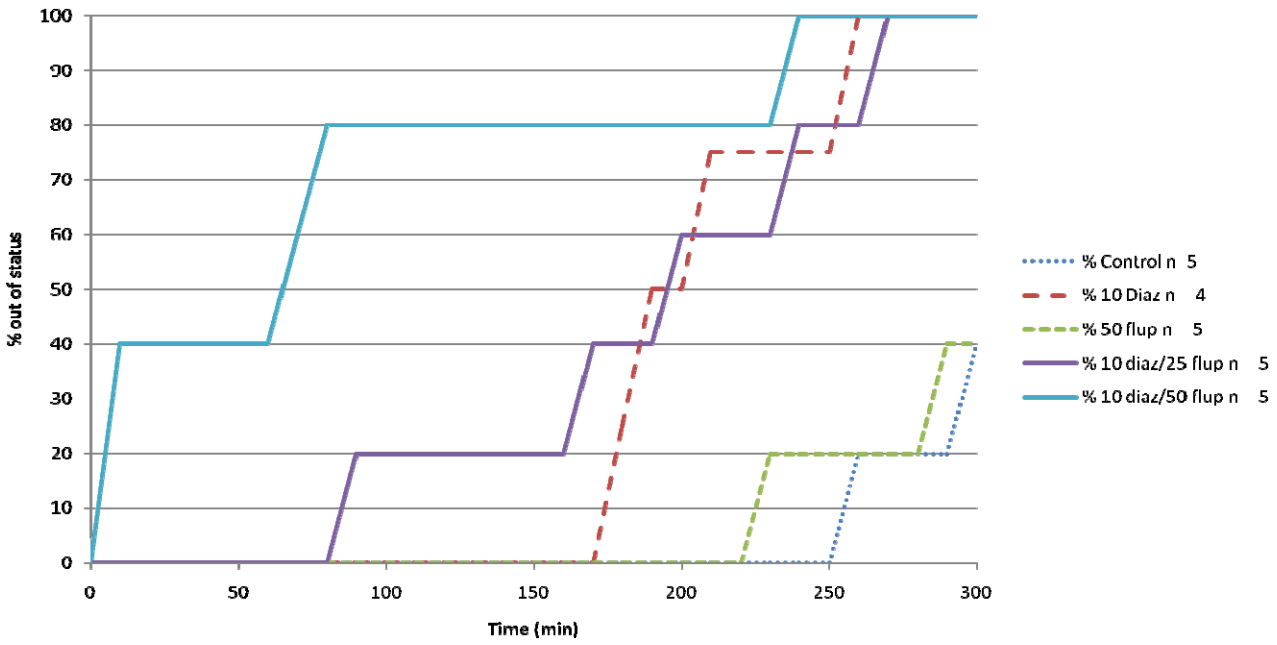



Figure 6. This demonstrates the Time-course of termination of status epilepticus (SE) following treatment with saline (controls), diazepam 10/mg/Kg, flupirtine 50 mg/Kg and 25 mg/Kg flupirtine combined with diazepam 10 mg/Kg and flupirtine 50 mg/Kg combined with diazepam. Note that the combination effectively terminated SE within 60 mionutes in 4/5 animals.

**A presynaptic action of M-type potassium channels in Schaffer-collateral CA1 pyramidal synapses**

Abstract

Muscarinic activation by its agonists such as pilocarpine and anologs results in seizures presumably by the inhibition of M currents. Muscarinic stimulation modulates glutamatergic synaptic transmission but the mechanisms remain uncertain. Studies were performed by whole-cell voltage and current patch-clamp techniques to test whether M currents modulate glutamatergic synaptic transmission and whether the drugs that open these channels could terminate seizures induced by muscarinic stimulation.

XE-991 (10 μM) an M channel blocker increased the frequency of spontaneous excitatory postsynaptic currents (sEPSCs), but had no effect on its amplitude. XE-991 and linopordine had similar effect on miniature excitatory postsynaptic currents (mEPSCs). They increased the frequency of mEPSCs but had no effect on its amplitude. M1 agonist NcN-A-343 also increased the frequency of mEPSCs. M channel opener flupirtine had the opposite effect from XE-991. The effect on XE-991 was eliminated by calcium free external ACSF. P/Q- and N-type of voltage-gated calcium channel blocker partially inhibited the effect of XE-991. These data suggest that M channel blockage increases presynaptic calcium dependent glutamate release in CA1 neurons.

In CA3 neurons, XE-991 increased bursting firing and depolarized membrane potentials. McN-A-343 had similar effect on CA3 neurons as XE-991. These data together suggest cholinergic activation inhibits M channels that cause presynaptic CA3 neuron depolarization, which activates voltage-gated calcium channels and elevates intracellular calcium concentration to increase glutamate release.

We tested whether M channel opener could terminate status epilepticus induced by lithium and pilocarpine. Administration of M channel opener flupirtine (50 mg/kg) and diazepam (10 mg/kg) had a less blockage, but administration both together had much stronger and faster blockage than them alone on cholinergic seizures in rats. 80% of rats were out of status in around 80 min's administration of flupirtine and diazapam. These data are consistent with cholinergic inhibition of M channel contribution seizure generation. Together, this study provides a novel mechanism of cholinergic seizures.

Key words: cholinergic seizure M channel glutamatergic synaptic transmission hippocampus